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Inhibitor-resistant early ethylene production during tomato fruit development

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Abstract – In addition to the ethylene formed at the onset of tomato fruit ripening, three peaks of ethylene are produced during earlier periods of in vitro development of tomato flower to fruit. This is the first report characterizing ethylene production during early development of tomato fruit. Previous reports from this laboratory showed that VFNT Cherry tomato calyces are transformed into fruit tissue when cultured in vitro at lower temperatures (16–23 °C). Early ethylene production was also measured in these ripening calyces, as well as in fruit and calyces of other tomato cultivars cultured in vitro. Calyces from Ailsa Craig and *rin* tomato flowers, which are not transformed into fruit tissue at these lower temperatures, also form ethylene during early periods of in vitro culture, but to a much smaller extent. Unlike ethylene formed at the onset of fruit ripening, the earlier peaks are resistant to the inhibitors, aminovinylglycine (AVG) and $CoCl_2$. The data suggest that ethylene produced during earlier periods of tomato fruit development is formed by an alternative biosynthetic pathway. © 2000 USDA-ARS. Published by Éditions scientifiques et médicales Elsevier SAS

Calyx / ethylene / fruit development / in vitro / tomato

ACC, 1-aminocyclopropane-1-carboxylic acid / AVG, aminovinylglycine [2-amino-4-(2'-aminoethoxy)-*trans*-3-butenoic acid] / SAM, S-adenosylmethionine

1. INTRODUCTION

The phenomenon of in vitro conversion of tomato calyces into fruit tissue has been investigated for several years since its discovery in this laboratory [14, 15]. Isolated VFNT Cherry tomato calyces separated from post-anthesis flowers or young fruit develop many of the characteristics of fruit tissue when cultured in vitro at temperatures slightly lower than normal (between 16 and 23 °C). They swell, lose their green color, and turn red and succulent. This phenomenon also occurs when the calyx is attached to the fruit. Coincidental to this physical transformation are a number of physiological changes that are characteristic of ripening fruit, including the production of flavor volatiles and sugars, and softening of cell walls.

Ethylene production during the ripening of many fruits has been known for some time [1]. Ethylene is also produced by virtually all parts of higher plants, acting as a regulator of a wide range of plant processes, such as seed germination, response to wounding, and senescence (see reviews [1, 8, 19, 30]). In the course of examining various ripening parameters, we measured ethylene production of intact tomato fruit, ovulary (fruit minus calyx), and isolated, ripening calyces. We discovered that, in all of these tissues, bursts of ethylene are produced, not only during ripening as the fruit turns from green to red, but also at several earlier phases of fruit development.

2. RESULTS

2.1. Ethylene production during early fruit development

Ethylene production and growth of VFNT Cherry tomato fruit, ovularies (fruit minus calyx), and calyces during in vitro development at 16 °C were measured for 63 d, beginning 3 d after initiation of culture (*figure 1*). As expected, a large increase in ethylene evolution was observed at the onset of fruit ripening,

around 40 d after anthesis. However, in addition to this well-documented ethylene production [1], fruit samples exhibited three bursts of increased ethylene production before fruit began to ripen (*figure 1 A*). An initial burst of ethylene occurred 5 d after initiation of culture (roughly equivalent to anthesis), with a subsequent decline in ethylene production. A second large burst occurred between 12 and 17 d and was followed by a third burst between 32 and 40 d. Ethylene and growth measurements of ripening VFNT Cherry tomato ovulary (fruit minus calyx) and calyx showed that these tissues behaved similarly to fruit tissue in all aspects of ethylene production (*figure 1 B, C*), but with slight shifts in peaks. In addition, these early bursts of ethylene seemed to coincide with changes in growth rate of the tomato tissues (*figure 1 A–C*). (Ethylene production in calyces was generally higher than or as high as in fruit, both during early development and ripening. This difference in ethylene evolution during ripening was noted previously [14].) Fruit and ovulary both showed a decrease in the rate of growth corresponding to the third ethylene peak; whereas calyx tissue showed an increase in growth rate at this time. The third peak of both ovulary and calyx is also much larger than that of intact fruit. In addition, the growth of ovulary cultures was considerably less than that of intact fruit.

In a previous report [14], we showed that ethylene production of ripening VFNT Cherry tomato calyces mimics that of ripening fruit, indicating a developmental change of the calyx to that of fruit. We also showed that in vitro tomato calyx ripening appears to be a unique phenomenon and, of the various tomato lines tested, occurred only in VFNT Cherry tomato. For example, culturing calyces from cultivars Ailsa Craig and *rin*, a ripening-inhibited line [22], at lower temperatures failed to induce their transformation into fruit tissue. It was, therefore, of interest to establish whether the occurrence of these early bursts of ethylene during in vitro development of tomato fruit and calyces represents another unique characteristic of VFNT Cherry tomatoes or is common to all developing tomato fruit tissue. Calyx samples of Ailsa Craig and *rin* were, therefore, cultured at 16 °C and the same parameters measured (*figure 2*). Calyces from both cultivars produced smaller amounts of ethylene than did calyces of VFNT Cherry tomato, but followed similar patterns of ethylene production – three bursts of ethylene formation were observed before the ripening phase. These calyces formed considerably less ethylene during the period at which ripening would occur in tomato fruit and VFNT Cherry calyces, and, as expected, the calyces did not develop into fruit. Again, a change in the growth rates of calyx tissues increased coinciding with or just after the third ethylene peak (not shown). Ethylene production of developing Ailsa Craig tomato fruit also followed the same pattern (data not shown).

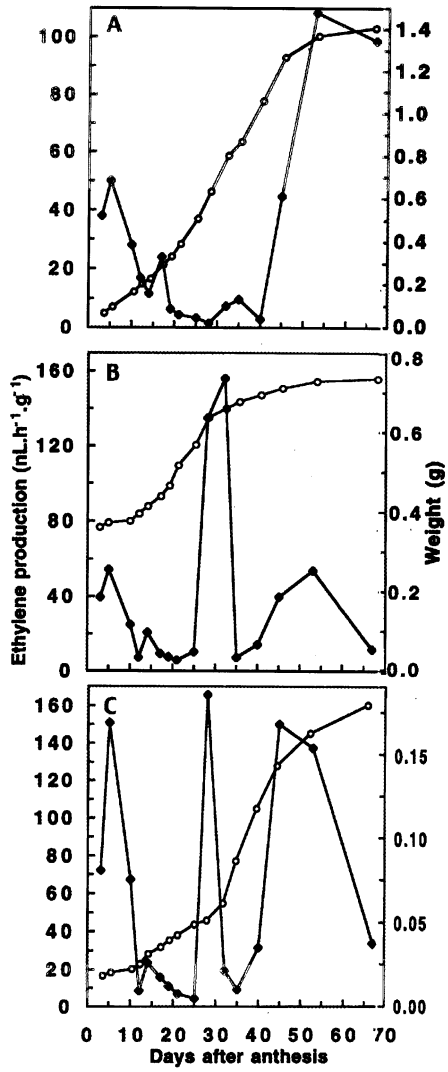

Figure 1. Ethylene production of VFNT Cherry tomato fruit, ovulary, and calyx cultures. Ethylene production (♦) and weight (○) were measured in VFNT Cherry tomato (**A**) fruit, (**B**) ovulary (fruit minus calyx), and (**C**) calyx in vitro. Experiments were performed in triplicate and repeated three times. The various bursts of ethylene produced by these plant organs varied slightly with regard to number of days after anthesis and magnitude. However, at least three early peaks always occurred before the large ripening-related peak, which occurred after 40 d.

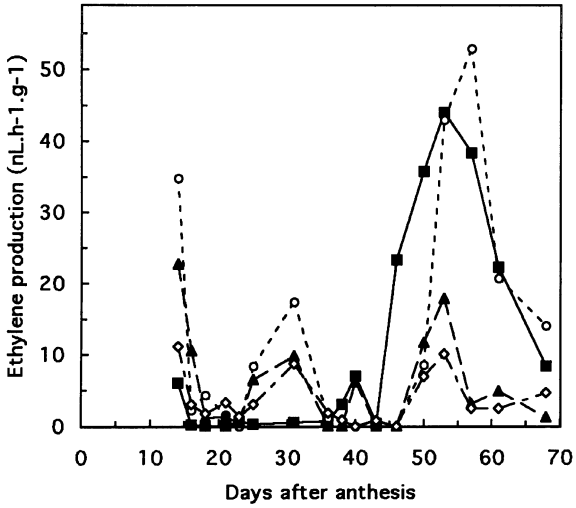

Figure 2. Ethylene production of VFNT Cherry tomato fruit (■) and calyx (○) and Ailsa Craig (▲) and *rin* (◇) calyces. This set of experiments was conducted early in our investigation. Ethylene measurements were not taken until 14 d post-anthesis. Therefore, the first burst of ethylene, whose maximum would be observed at approximately 5 d, is not recorded. The experiment was conducted in triplicate.

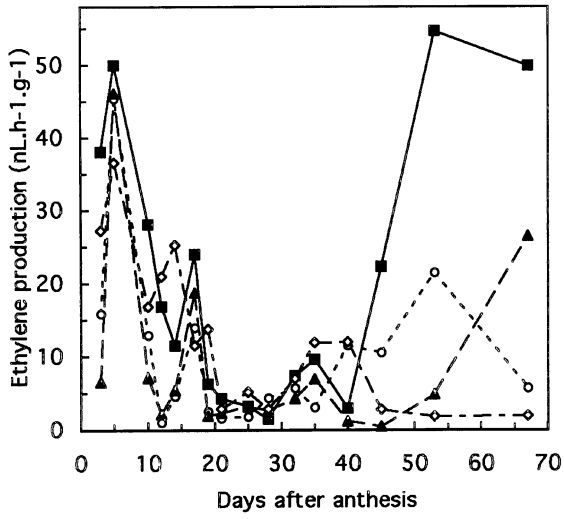

Figure 3. Effects of various AVG concentrations on ethylene production by VFNT Cherry tomato fruit. AVG concentrations tested were 10 µM (○), 50 µM (▲), and 100 µM (◇). Ethylene formed by fruit cultured in the absence of AVG (■) is given as a control, and these values are connected by the solid line. Experiments were conducted in triplicate and repeated twice.

The time course of ethylene peaks was not identical in all experiments, and the magnitude of ethylene production varied. However, the number of peaks and the change in growth rates were consistent in the four experiments conducted, each using triplicate samples of each tissue.

2.2. Effects of inhibitors on ethylene production during early fruit development

2.2.1. Aminoethoxyvinylglycine

Aminoethyoxyvinylglycine [2-amino-4-(2'-amino-ethoxy)-*trans*-3-butenoic acid (AVG)], an inhibitor of ripening-related ethylene production [3–6, 11, 31], was added at three concentrations (10, 50 and 100 µM) to the medium in which either VFNT Cherry fruit and calyces were cultured in vitro (*figure 3*). This range covered concentrations found effective in ethylene inhibition studies in which apples were sprayed [6] or tomato and avocado fruit slices were incubated in solutions containing AVG [5]. Ethylene production decreased with increased AVG concentration until, at 100 µM AVG, fruit growth was limited (not shown), ethylene production was almost completely inhibited, and fruit no longer ripened. However, AVG did not have a similar inhibitory effect on ethylene bursts during earlier periods of development. In fact, in the experiment shown in *figure 3*, the second and third bursts of ethylene increased with increased AVG concentration. However, this increase was not always observed. Similar results were obtained in experiments with calyces but are not shown.

2.2.2. Cobaltous ion

Cobaltous chloride, which was shown to inhibit ethylene production in mung bean hypocotyl [18] and post-climacteric, vacuum-infiltrated apple fruit [18, 31], was added to the culture medium at three concentrations (50, 100 µM and 10 mM) to determine its effects on ethylene production by tomato fruit and calyces (see *figure 4*). These concentrations are similar to those that were shown previously to inhibit ethylene production effectively [18, 31]. Data from calyces are not shown, but were very similar. Once again, ethylene production during fruit ripening was increasingly inhibited with increasing concentrations of $CoCl_2$. At 10 mM $CoCl_2$, fruit growth was greatly impaired (not shown), ripening-associated ethylene was completely inhibited, and fruit failed to ripen. Early bursts of ethylene, however, persisted and, even at the highest concentration of Co^{2+}, were almost as high as corresponding peaks of ethylene production in control fruit.

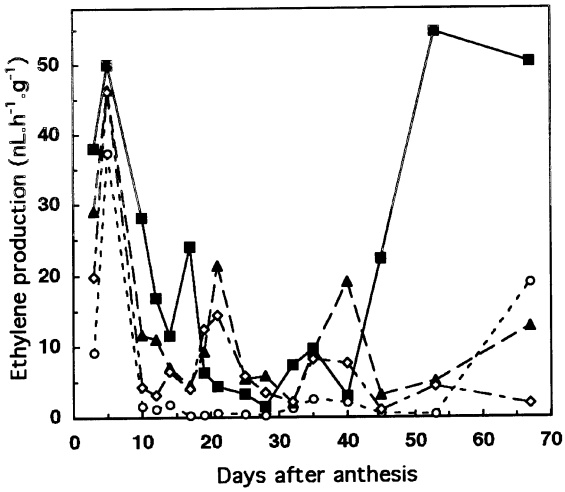

Figure 4. Effects of various Co^{2+} concentrations on ethylene production by VFNT Cherry tomato fruit. Concentrations of $CoCl_2$ tested were 50 µM (○), 100 µM (▲), and 10 mM (◇). Ethylene evolution from fruit cultured in the presence of these concentrations of Co^{2+} was compared with that produced by fruit cultured in the absence of Co^{2+} (■). Control values are connected by a solid line. Experiments were conducted in triplicate and repeated twice.

3. DISCUSSION

The biosynthetic pathway of ethylene in higher plants has been firmly established for some time and has been reviewed extensively (see for example, [1, 8, 16, 19, 30]). Ethylene is formed from methionine by the following steps (see *figure 5*): S-adenosylmethionine (SAM) is synthesized by the action of SAM synthase (EC 2.5.1.6). ACC synthase (EC 4.4.1.14) then converts SAM to 1-aminocyclopropane-1-carboxylic acid (ACC), which is converted enzymatically to ethylene by the action of ACC oxidase.

AVG is a potent inhibitor of ethylene biosynthesis, both in vitro and in vivo [3–6, 11, 31]. It is an inhibitor of pyridoxal phosphate-mediated reactions [4] and inhibits the conversion of SAM to ACC by ACC synthase [3] in the formation of ethylene (*figure 5*). AVG is effective in reducing ethylene production in higher plants, but generally does not abolish it [1–5, 31]. Baker et al. [5] found that AVG inhibited both total ethylene production and [^{14}C]-methionine incorporation into ethylene more completely in green than in pink tomato fruit tissues. For example, at one inhibitor concentration, ethylene production was inhibited by 69 % in mature green tomato slices, but only 11 and 13 % in climacteric (pink) and post-climacteric (red) fruit, respectively. The authors suggested that this difference in sensitivity to AVG might be explained by competitive action of a larger methionine pool for ethylene formation in the riper tissue. They stated, however, that the possibility of an alternate pathway of ethylene formation (one that uses methionine, but differs in sensitivity to AVG), a change in enzyme sensitivity to AVG, or altered uptake or metabolism of AVG could not be ruled out. This resistance to AVG is also indicated in the present experiments. The average inhibition of ethylene evolution obtained in tomato fruit at the highest AVG concentration used was 95 % (range: 90.2–96.6 %) (see *figure 3*). Concentrations greater than this would have been toxic to fruit tissue.

$CoCl_2$ inhibits the conversion of ACC to ethylene, the last step of ethylene biosynthesis [18, 31] (*figure 5*). In our experiments, the ethylene production of ripening tomato fruit was almost completely inhibited by 10 mM $CoCl_2$, but the early peaks of ethylene formation persisted. At this Co^{2+} concentration (10 mM), the first peak was as high or higher than those at lower inhibitor concentrations.

Ethylene production during early fruit development is not unexpected and has been reported by several investigators, but not characterized [17, 28], and the bursts of ethylene may signal the initiation of molecular events that regulate fruit development. A number of phenomena occur that could be associated with ethylene production. High rates of ethylene production in

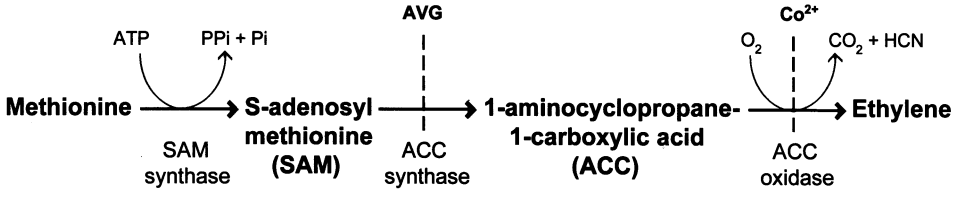

Figure 5. Biosynthesis of ethylene in higher plants.

fruit are associated with rapid growth and cell division and may reflect high auxin concentrations [28]. In fig fruit [21], ethylene inhibits cell division during the early period of fruit growth and promotes cell expansion and fruit growth later. In tomato fruit, after approximately 7–14 d, cell division stops, and, thereafter, fruit size increases as a result of cell expansion [7, 9]. The cessation of cell division and beginning of cell expansion coincide with the appearance of the first peak of ethylene production and an increase in growth rate of fruit in our experiments. Lacheene and El-Beltagy [17] showed that, in tomato fruit, auxin levels reached a maximum 9 d after anthesis and after 20 d were not measurable. Auxin stimulates ethylene production by inducing ACC synthase, which converts SAM to ACC [31]. Because precursors of ethylene are also precursors of other biosynthetic pathways and are involved in many physiological processes, fluctuations in ethylene production during development would be expected. SAM, for example, is a precursor in both ethylene and polyamine biosynthesis [12] and is also involved in DNA methylation [27].

The production of ethylene in response to wounding is documented in numerous species of plants [1]. Generally a lag period of about 20 min to 1 h is followed by a large, rapid increase in ethylene evolution. Likewise wounding induces increased mRNA and activity of ACC oxidase [20] with a time course of several hours. Maximum ACC oxidase mRNA levels were reached in 3 h and diminished to pre-wounding levels within 24 h. Therefore, the first peak of ethylene production reported here, which has its maximum at 5 d, is very unlikely to be explained as a result of wounding from excising tissues during culture.

It is also of interest to note in *figure 2* that ethylene production of calyces from Ailsa Craig and *rin* tomato fruit is much smaller during the period corresponding to ripening of VFNT tomato calyces. This is expected since no apparent change has taken place in the developmental program from that of calyces to that of fruit. However, a smaller peak of ethylene production does occur at the time coinciding to fruit ripening. In addition, earlier peaks of ethylene are produced by calyces from both cultivars. This indicates that the potential for conversion of the calyx to fruit is present in these cultivars, but part of the mechanism, e.g. activation of carotenoid biosynthesis, is missing.

Discrepancies in total growth and in changes in growth rates of fruit, ovulary, and calyx in these experiments are of interest. Final size of the ovulary is much less than that of fruit, which has been observed previously [10, 24] and indicates that the calyx contributes something essential to ovulary growth. In addition, calyx growth rate increases at the same time as, or just after, the third ethylene peak, unlike fruit and ovulary tissue, which decrease. This difference might be related to the developmental conversion of calyx to fruit tissue.

The present experiments indicate that ethylene production in developing tomato fruit is more complex than previously described. Earlier data on fruit development describe a large burst of ethylene coinciding with the period when fruit begins to ripen. In tomato, this occurs as the fruit begins to turn from greenish yellow to red, the breaker stage. In addition to this ripening-related ethylene production, our experiments clearly demonstrate three earlier, previously uncharacterized, bursts of ethylene, well before the onset of ripening (before breaker stage). These earlier periods of ethylene production differ from the ripening-related production by their insensitivity to inhibitors of two separate steps of the established ethylene biosynthetic pathway: AVG, which inhibits ACC synthase in the conversion of SAM to ACC, and $CoCl_2$, which inhibits ACC oxidase and the enzymatic conversion of ACC to ethylene. These inhibitors are effective in greatly reducing the ripening-associated ethylene production in tomato fruit tissue. However, at the same concentrations, the early peaks of ethylene production that are probably associated with molecular events that are essential to fruit development remain virtually unaffected. In fact, the magnitude of the earlier peaks is not apparently related to inhibitor concentration, and peaks are sometimes shifted slightly by the presence of inhibitors. Our data are consistent with an alternate pathway of ethylene biosynthesis to explain ethylene formation during early fruit development. An alternate pathway for ethylene synthesis has also been indicated in the epidermal cells of leaves of a pea mutant Argenteum, as well as in lower plants (liverworts, mosses, ferns, Gnetales, cycads, gymnosperms, and angiosperms) [26].

4. METHODS

4.1. Plant material

Ovularies and calyces of greenhouse-grown tomato plants (*Lycopersicon esculentum*, cv. VFNT Cherry) were cultured in vitro [25], starting with post-anthesis flowers or small, green fruit (< 5 mm). Plant material was wrapped in several layers of cheesecloth, disinfested by immersing for 10 min in a saturated calcium hypochlorite solution containing Tween 20 (polyoxy-

ethylene sorbitan monolaurate, Sigma Chemical Co.) (two drops 100 mL^{-1}), and then rinsed three times in autoclaved, deionized water. Ovulary cultures (for fruit samples) were prepared by removing corolla, stamen, and most of the pedicel from the flower. The pedicel of a single flower (ovulary) was then inserted into a hole made in the center of a filter paper platform [13]. The platform was then placed on the surface of a medium containing Murashige and Skoog [23] salts, sucrose (0.175 M), myo-inositol (0.555 M), and White's [29] vitamins and glycine, at pH 5.7. Stock solutions of AVG (Sigma Chemical, Co., St. Louis, MO) were prepared, filter-sterilized using a 0.2-µm Acrodisc filter (Gelman Sciences, Ann Arbor, MI), and small volumes added to individual culture tubes containing hot, sterilized medium. Small amounts of a stock solution of $CoCl_2$ were added to the medium before sterilizing in an autoclave. Calyces from flowers or small, green tomato fruit (< 5 mm) were cultured separately, by placing the calyx from a single flower or fruit on the surface of the solidified medium. Care was taken so that the surfaces of the calyx made good contact with the culture medium. Cultures were kept in a growth room either at 16–17 °C and illuminated by Grolux lamps (Sylvania, Inc. Danvers, MA) at an average photosynthetically active radiation of 27.7 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (range = 21–31) for 16 $h \cdot d^{-1}$.

4.2. Ethylene measurements

Ethylene produced by tissues was collected by sealing culture tubes, using sterilized rubber septa, for approximately 1 h at room temperature (23 °C). Gas samples were injected into a Hewlett-Packard gas chromatograph, Model 6890, equipped with a 1/4-inch stainless steel column (Porapak-N, 80 to 100 mesh, Alltech Associates, Inc., Deerfield, IL), a flame ionization detector, and an HP 3396 Series II integrator (Hewlett-Packard). The instrument was calibrated, using ethylene gas mixtures (ethylene in helium) obtained from Scott Specialty Gases (Fremont, CA). Plant tissues were weighed under aseptic conditions, using an OHAUS balance, Model GT210, in a laminar-flow hood after each series of ethylene measurements.

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